Ferritin Crystal Cataracts in Hereditary Hyperferritinemia Cataract Syndrome

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PURPOSE. Hereditary hyperferritinemia cataract syndrome (HHCS) is a genetic disease defined by cataracts, hyperferritinemia, and ferritin light-chain (L-ferritin) gene mutations. HHCS was diagnosed in this study in one of the first families known to be affected in the United States, and the basis of lens opacities in HHCS was determined.

METHODS. DNA amplification and sequencing of the human L-ferritin gene was used for mutation detection. RNA electrophoretic mobility shift analysis was performed to demonstrate functional consequences of a new mutation. Opacities were characterized by immunohistochemical and electron microscopic analyses of human HHCS lens aspirates.

RESULTS. HHCS was diagnosed in five members of one family who had all three hallmark features: hyperferritinemia, a prominent cataract history, and the finding of a novel mutation in the L-ferritin gene (C337T). This mutation interferes with function of the L-ferritin transcript in an RNA gel shift assay. Light-diffracting crystalline deposits were present in cataractous lenses from two affected family members but not in control lenses. Immunohistochemical analysis showed strong anti-L-ferritin reactivity in the crystalline deposits. Analysis of these deposits by transmission electron microscopy with fast Fourier transformation demonstrated macromolecular crystalline structure of the deposits. The data were consistent with a face-centered cubic crystal having a unit crystal cell size of 17 nm, both findings characteristic of ferritin crystals grown in vitro.

CONCLUSIONS. HHCS cataract is due to numerous small opacities, predominantly in the lens cortex, that are light-diffracting ferritin crystals. Patients with HHCS may be recognized by a family history of cataracts and hyperferritinemia without increased serum iron. (Invest Ophthalmol Vis Sci. 2002;43: 1121–1126)

Cataracts are the predominant clinical abnormality in the recently described genetic disease hereditary hyperferritinemia cataract syndrome (HHCS).1–3 HHCS has been identified in patients who had both high serum ferritin (hyperferritinemia) and a prominent history of bilateral, early-onset cataract.1–2 It is inherited in an autosomal dominant manner1,2 and results from mutations that cluster in a discrete regulatory region of the human L-ferritin gene, as discussed later in the article.4–5 A 5- to 20-fold increase in ferritin is found in serum, lymphocytes, liver, aqueous humor and lens of patients with HHCS.1–3 Recent data indicate that patients with HHCS have ferritin accumulations in other tissues and that this may have pathologic consequences (Brooks D, Stambolian D, manuscript in preparation).

HHCS is caused by a novel mechanism involving loss of the normal regulation of ferritin gene expression. The mechanism by which iron regulates ferritin synthesis is a paradigm of translational regulation of gene expression (reviewed in Ref. 6). The two essential components of this regulatory system are a cis-acting iron-responsive element (IRE) and trans-acting iron-regulatory proteins (IRPs) as shown in Figure 1. The IRE in ferritin mRNAs is a hairpinlike structure near the beginning of the 5′ untranslated region (UTR). IRPs bind this RNA element and decrease ferritin translation by competing for ribosome binding. An increase in iron decreases IRP binding, relieving inhibition of ferritin mRNA translation (Fig. 1, middle panel), resulting in increased ferritin storage of iron. This iron-dependent regulation of ferritin expression was observed in vitro, but its significance was confirmed by the identification of HHCS, in which aberration of IRE-mediated regulation of ferritin synthesis leads to persistent hyperferritinemia (Fig. 1, right panel).4,5

Ferritin is found in the lens, where it may have a role in iron homeostasis, as it does in other tissues.7 Assembly of L (light chain) and H (heavy chain) ferritin subunits into a 24-subunit complex yields apoferritin. Apoferritin is a cage-like structure capable of containing up to 4000 atoms of iron as holoferritin. H-ferritin translocates and oxidizes ferrous iron inside the ferritin cage. L-ferritin, which does not bind iron directly, facilitates mineralization of ferric iron inside the cage to form holoferritin. The coordinated action of L- and H-ferritin subunits detoxifies ferric iron inside the holoferritin cage, thereby limiting redox cycling of iron and generation of reactive oxygen species. These functions of ferritin, iron storage, and detoxification, did not provide an obvious hypothesis connecting overexpression of L-ferritin to cataract. The present study was undertaken to diagnose HHCS and determine the cause of cataract in this disorder.

Cataracts may appear as early as infancy in HHCS and have been described as numerous, pulverulent (dustlike) opacities of the lens2 that cause glare and decrease visual acuity.9 Two groups have reported a 5- to 10-fold higher L-ferritin content in HHCS lens and/or aqueous humor than in non-HHCS eyes.9–9 However, their findings differed regarding the form of L-ferritin in lens. One group reported only soluble ferritin,3 whereas another group suggested that L-ferritin forms crystalline-appearing deposits in the lens.7 Herein, we report a novel mutation of the L-ferritin IRE and demonstrate that it has functional consequences that result in HHCS. Comprehensive evaluation of lens specimens removed during cataract surgery demonstrates that light-diffracting crystals of L-ferritin-rich isoferritin are present in the lens of patients with HHCS. We argue that...
these crystals are one type of opacity causing cataract in HHCS and an unprecedented example of a wild-type protein's crystallizing in human lens.

**METHODS**

**Family Data**

A 50-year-old woman sought treatment at the Hemochromatosis Clinic at the University of Pennsylvania because of a personal and family history of hyperferritinemia. Medical history included stable ferritin levels of 1300 to 1440 ng/mL (normal: 50–200) with normal serum iron, transferrin, transferrin saturation, and HFE-1 mutation testing. Bilateral cataracts were diagnosed at 5 years of age; progressive glare prompted bilateral cataract surgery in her fourth decade. This proband and five family members were subjected to ophthalmic examination, serum ferritin assay, and human L-ferritin IRE genotyping. Four additional family members were found to have hyperferritinemia in the range of 969 to 1350 ng/mL. Each of these individuals also exhibited cataracts—numerous small opacities, predominantly in the cortex of each lens. One affected family member had received a previous misdiagnosis of hemochromatosis and prescribed phlebotomy therapy. Removal of 2 units of blood over 3 weeks induced symptomatic iron-deficiency anemia, a characteristic response in patients with HHCS to inappropriate phlebotomy. Two family members with normal serum ferritin had clear lenses. The one who was available for genetic testing had a normal L-ferritin IRE DNA sequence. All patients signed informed consent. This research protocol was approved by the Institutional Review Board of the University of Pennsylvania and conformed to the tenets of the Declaration of Helsinki.

**DNA Sequence Analysis**

Peripheral blood leukocyte genomic DNA was prepared from individuals with hyperferritinemia who had cataract, and control DNA was collected from 65 cataract-free individuals (150 chromosomes). The human L-ferritin gene was subjected to PCR amplification, using oligonucleotides and conditions as described in Girelli et al. PCR amplicons and individual subclones were sequenced by dideoxy-termination (ABI 377 sequencer; PB-Applied Biosystems, Foster, CA).

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**Figure 1.** Schematic representation of IRE-IRP status in various clinical conditions, including the consequences of changes in IRE-IRP on ferritin translation and physiology. *Left:* in iron deficient cells, IRP was bound to IRE in the 5'UTR of L-ferritin mRNA. In this position, IRP inhibited binding of the small ribosomal subunit to ferritin mRNA and therefore inhibited translation. This resulted in a decrease in ferritin synthesis, thereby shifting iron from storage to bioavailability. *Middle:* at high iron concentrations, IRP was not bound to IRE. IRP1, as shown, contains an Fe-S cluster that occupies the RNA binding site and IRP2 was degraded in high iron conditions (not shown). Without IRP bound, there was no inhibition of ferritin translation, and iron was stored in the newly synthesized ferritin. *Right:* in HHCS, mutation of the IRE (illustrated as a change in the shape of stem of the IRE) decreased the affinity for IRP and resulted in constitutive, iron-independent translation of ferritin mRNA and hyperferritinemia.
Human Lens Preparation

Two affected individuals had cataract surgery that produced a disrupted lens aspirate in saline suspension. This aspirate was centrifuged, and the pellet was examined directly (untreated) as well as fixed in 10% formalin or 1% glutaraldehyde and embedded in paraffin or Epon, respectively. The inclusion of formalin or glutaraldehyde in the fixation procedure is essential for preservation of cataractous deposits, which are often imperceptible in routine hematoxylin and eosin staining. Electron micrographs were obtained by a transmission electron microscope (TEM; model 2000; JEOL, Tokyo, Japan) operating at 80 kV. Specimens were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, immersed in 1% uranyl acetate, and then stained in 1% uranyl acetate before dehydration in graded acetone and embedding in Epon. Ultra-thin sections were cut and stained with lead citrate. Images were digitized to produce 2048 x 2048 eight-bit gray-scale images. The Fourier transforms of the digitized images were calculated to identify characteristic length scales translation vectors within the images.

RNA Electrophoretic Mobility Shift Assay

A 289-nucleotide amplicon containing the first 180 nucleotides of the human L-ferritin 5' UTR was prepared as described by Girelli et al. and cloned into the pCR2.1 vector containing T7 RNA polymerase promoter (Invitrogen, San Diego, CA). In vitro transcripts were generated from this construct and used for electrophoretic mobility shift assay. IRP activity was detected in this cell line by its ability to produce a band shift when the probe is incubated with cytosolic extracts. The amount of active IRP was determined by densitometry of the bands in a gel electrophoresis gel. The gel was stained with Coomassie Blue to visualize the lanes and the bands. The densitometry was performed using a scanning densitometer. The intensity of the bands was compared to a standard curve to estimate the amount of active IRP.

RESULTS

A Novel HHCS Mutation

Members of a family cosegregating hyperferritinemia and cataract were screened for mutations in the L-ferritin IRE by PCR amplification and DNA sequencing. A novel heterozygous mutation, C33T, was found in the DNA that replaces the unpaired cytosine in the stem of the IRE with uracil (C33U in DNA) in the L-ferritin mRNA (not shown). This change was found in all five affected family members and was absent in an unaffected family member. Analysis of 130 control chromosomes (cata
cract-free individuals) did not detect the C33T change. An exclusively wild-type IRE sequence was found in all control specimens.

IRP Activity in Human Lens Epithelial Cells and the Effect of the C33U Mutant IRE

To determine whether the C33U mutation of the L-ferritin IRE has functional consequences on IRP binding and on cataract formation we sought to demonstrate that IRP exists in human lens cells. The RNA electrophoretic mobility shift is the assay of choice for IRP function, because IRP bound to IRE-containing RNAs shifts the RNAs in electrophoreses. A cytosolic extract prepared from the human lens epithelial cell line HLEB5, as a source of IRP activity, was incubated with radioactive probe from the human L-ferritin 5' UTR. Arrow: position of a band indicating slow-migrating and nondegraded probe, only when the input RNA is of a sense orientation and therefore capable of forming an IRE hairpin. Sense-strand RNA probes of wild-type and mutant (C33U) sequences are compared for their ability to bind IRP and produce a gel shift. As shown in both lanes, labeled mutant, neither fibroblast (F) nor lens (L) cell IRP did not bind to and retard migration of the mutant RNA probe, because of decreased affinity. This results in the probe's being degraded.

Ferritin Crystalline Opacities are Cataracts

These deposits diffract light, as evidenced by the diffraction lines and the frequent, regular, polyhedral, crystalline-appearing deposits (Fig. 3B). Crystalline-Appearing Deposits in HHCS Lens

These deposits produce a band shift (Fig. 2B). These results indicate that the C33U mutation impairs binding of the mutant RNA to IRP.

Crystalline-Appearing Deposits in HHCS Lens

In HHCS, cataracts consist of numerous, small, punctate opacities predominately in the cortex of the lens, as shown in Figure 3A. Clinically, these cataracts cause progressive glare and impair visual acuity. Light microscopy of a fresh HHCS lens specimen demonstrated frequent, regular, polyhedral, crystalline-appearing deposits (Fig. 3B). These deposits diffract light, as evidenced by the diffraction lines adjacent to the long axis of a deposit (Fig. 3B, arrows). H&E staining revealed uniform cosinophilia of the deposits (Fig. 3C) which also showed strong staining with Masson trichrome (not shown). Congo red produced only background staining of deposits (not shown). De-
was faint anti-L-ferritin immunoreactivity in a minority of lens dense immunoperoxidase reactivity of the deposits (Fig. 4B). There clonal antibody LF03, directed against human L-ferritin, produced comparable titer revealed no staining of the deposits (Fig. 4A). Monotoxination of deposits in HHCS lens. Control antibody (isotype-matched) at L-Ferritin in Crystalline-Appearing Deposits in posits typically measured 15 to 20 μm wide by 40 to 150 μm long. Low-magnification TEM of deposits demonstrated linear external borders with a dense central area surrounded by a less-dense periphery (Fig. 3D). Prominent ultrastructural features of these deposits were linear defects, or areas of low electron density, the longest of which traversed the deposit (Fig. 3D). These defects had nonrandom orientation and were typically linear and parallel to an exterior face of the deposit (Fig. 3D). Therefore, HHCS lens contained deposits that had histologic staining characteristics of protein with physical and morphologic properties, suggesting a highly ordered structure. No such geometric-appearing deposits were observed in four lens specimens removed and processed under identical conditions from patients with non-HHCS cataracts (not shown).

**L-Ferritin in Crystalline-Appearing Deposits in HHCS Lens**

Immunostaining was performed to determine the antigenic composition of deposits in HHCS lens. Control antibody (isotype-matched) at comparable titer revealed no staining of the deposits (Fig. 4A). Monoclonal antibody LF03, directed against human L-ferritin, produced dense immunoperoxidase reactivity of the deposits (Fig. 4B). There was faint anti-L-ferritin immunoreactivity in a minority of lens fibers outside the deposits (Fig. 4B). Anti-H-ferritin monoclonal antibody (rH02) staining of HHCS lens gave a pattern that was indistinguishable from that of control antibody (not shown). Both this anti-H-ferritin and the LF03 anti-L-ferritin monoclonal antibodies were strongly reactive with human liver under identical conditions (not shown). Therefore, the deposits contained levels of H-ferritin below the limit of immunodetection, or the H-ferritin epitope was not accessible to the antibody in the deposit.

Rabbit sera raised against human holoferritin were used to stain sections prepared for immunoelectron microscopy. Preimmune sera produced low-background staining of all lens tissue, including deposits (Fig. 4C). By contrast, electron-dense areas of deposit were strongly reactive with anti-ferritin sera (Fig. 4D). As observed at the light microscopic level there was sparse anti-ferritin staining of lens tissue outside the deposits (not shown). Together, these immunostaining results demonstrate that the crystalline-appearing deposits in HHCS lens were rich in the L-ferritin protein that was overexpressed in HHCS and contained little H-ferritin.

**Crystalline Structure and Composition of HHCS Lens Deposits**

Because iron is electron dense, ferritin-bound iron may be observed by the electron microscope. Indeed, this has formed the basis for the use of ferritin-conjugated antibodies as markers in immunoelectron microscopy for many years. Therefore, unstained (metal-free) electron microscopic sections containing the ferritin-rich deposits were examined, and no electron dense material suggestive of iron was observed (not shown). Electron-dispersive spectroscopy is capable of detecting metals in biological specimens. Application of this technique to lens deposits did not detect an iron signal above background, suggesting that the ferritin in lens deposits is not iron rich (data not shown). To investigate structure within HHCS lens deposits, TEM and fast Fourier transformation analysis of the resultant images was performed on a stained section of cataract tissue. TEM revealed regions within the lens deposit that show regular, periodic variations in image contrast (Fig. 5A). Fast Fourier transformations of these regions clearly indicated periodicity in at least two directions (Fig. 5B), indicating a macromo-
The second postulated mechanism invokes a well-known cause of cataract formation: loss of lens protein solubility. By this hypothesis, the overexpressed ferritin directly forms deposits or insoluble aggregates that are lens opacities. Ferritin-rich deposits with the appearance of crystals have been reported to be cataracts in HHCS.9 We have extended this analysis to demonstrate that the cataracts are light-diffracting crystals composed of iron-poor, L-ferritin-rich, and H-ferritin–poor isoferroin. The evidence is as follows: First, deposits were found in HHCS lens that are 3 to 10 lens fiber diameters across (Fig. 5C). This size was sufficient to disrupt packing of lens fibers (Figs. 4A, 4B) and was predicted to disrupt light transmission through the lens. Second, these deposits had a striking geometric (i.e., polyhedral) appearance (Figs. 3B–D, 4A, 4B). Third, internal linear defects in the deposits were geometric and nonrandom in structure (Fig. 3D). Fourth, the crystalline–appearing deposits diffracted light (Fig. 3B). Fifth, polyclonal and monoclonal antibodies against human ferritin densely stained the lens deposits (Fig. 4). Sixth, periodicity was evident in at least two directions in the deposits, as demonstrated by fast Fourier transformation of transmission electron micrographs (Fig. 5), indicating crystalline structure. The fast Fourier transform is consistent with a face-centered cubic crystal structure that is one of the crystal structures adopted by purified ferritin crystallized in vitro.12,13 Finally, the 17-nm unit cell size in this crystal is consistent with that of isoferroin crystals grown in vitro with an intermediate size between that expected for a wet isoferroin crystal (18.4 nm) and that of an air-dried ferritin crystal (15.8 nm).12,13 Together, these data confirm the presence of L-ferritin crystals in HHCS lens. We argue that L-ferritin crystals are the lens opacities that cause HHCS cataracts, because these crystals both diffract light and disrupt lens fiber packing and therefore focusing of light.

Cataract formation is the most prominent and only consistent clinical abnormality in HHCS. Because the gene is ubiquitously expressed, dysregulation of L-ferritin expression in HHCS is predicted to occur in most if not all tissues. Indeed, overexpression of L-ferritin in HHCS has been demonstrated in serum,1,2 liver,4 lymphoblasts3 and the eye (aqueous and vitreous humors and lens).5 Why might L-ferritin overexpression have a propensity to affect lens more than other tissues? At increased concentration, L-ferritin may cause cataracts because of properties of lens physiology that may promote protein crystallization. First, there is a very high-protein concentration in lens. Second, there is little protein turnover after formation of mature lens fibers. Lens proteins have half-lives measured in decades. Third, lens is avascular and surrounded by a dense
capsule; therefore, ferritin synthesized within the lens may not diffuse or be diluted out. Thus, when abnormally high L-ferritin concentrations are produced in HHCS lens, conditions such as time, low protein turnover, and high protein concentration are likely to contribute to ferritin crystallization. These features of lens biology may account for the absence of reported phenotypic changes in other tissues.

Crystal formation in the path of light transmission through the eye is inherently problematic, due to light diffraction. A single case of crystal formation in lens, as a sole cause of cataract, was reported previously, but the substance of the crystals remains unknown. Crystallization of calcium oxalate in lens has been reported in association with complicated cataract. Recently, protein crystals have been found to be cataracts for the first time. Specifically, two different mutations in human lens crystallin genes cause striking crystalline cataracts. Unlike HHCS, wherein the crystallized L-ferritin is of wild-type sequence, the proteins causing crystalline cataract in these instances are mutant. In one case, the mutant amino acid creates a salt bridge proposed to stabilize crystal structure.

Analysis of lens removed from two patients with HHCS during cataract surgery has provided important insight into the basis of cataract in this disease. The HHCS lens contains frequent crystals that are sufficiently large to disrupt packing of lens fibers and therefore the focusing of light by the lens. Subunits of these crystals have the size, crystallization characteristics, and antigenic properties of L-rich iso-ferritin. Physical appearance and properties, such as polyhedral shapes and periodic electron density within the deposit, demonstrate the crystalline nature of these L-ferritin deposits. This is the third example, to our knowledge, of protein crystals as a cause of cataract and may well represent the most common example, because more than 50 families with HHCS are known (data not shown). Future research will address the prevalence of HHCS, natural history of crystal formation, the implications of ferritin insolubility outside the eye in HHCS and other diseases, and potential therapeutic measures for crystal cataract diseases such as HHCS.

Acknowledgments

The authors thank the patients for their willing participation in this study; the late Patricia Grimes, Francis Munier, Carole Beaumont, Domenico Girelli, and Sonia Levi for helpful discussions; and Usha Andley for the HLEB3 cell line.

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